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Morphogenesis of Maternal and Paternal Genomes in Fertilized Oyster Eggs (*Crassostrea gigas*): Effects of Cytochalasin B at Different Periods During Meiotic Maturation

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Abstract. Fertilized oyster (*Crassostrea gigas*) eggs, treated with and without cytochalasin B (CB) at varying periods during meiotic maturation, were examined following 7-aminoactinomycin D (7-AAD) and anti-β tubulin staining for DNA and microtubular patterns, respectively. Electron microscopic observations of untreated fertilized eggs revealed the development of an extensive network of endoplasmic reticulum during germinal vesicle breakdown. Germinal vesicle breakdown was not accompanied by the disappearance of the nucleolus; the latter persisted morphologically unchanged throughout fertilization. The first meiotic spindle [12.4 ± 1.0 μm (l) × 5.4 ± 0.55 μm (diameter (d); metaphase plate)] was oriented with its long axis perpendicular to the egg's surface. In contrast, the second meiotic spindle was approximately one half the size of the first [7.4 ± 0.65 μm (l) × 2.91 ± 0.29 μm (d)] and initially oriented with its long axis parallel to the egg's surface. Just prior to anaphase II, the spindle rotated so that its long axis became perpendicular to the egg's surface. Following its incorporation into the egg cytoplasm, the sperm nucleus dispersed but did not form a nuclear envelope until the completion of polar body formation. Just prior to pronuclear migration, an array of microtubules assembled around the female pronucleus, and then regressed; concomitantly, a sperm aster formed in conjunction with the centrosome associated with the developing male pronucleus. Following their migration and apposition with one another, both pronuclei underwent prophase as independent structures. Chromosomes from the male and female pronuclei became organized on a mitotic spindle in preparation for first cleavage. In zygotes treated with CB continuously or for just meiosis I, anaphase I occurred; however, a polar body failed to form. Consequently, all the diads were retained within the zygote and became situated on a tripolar spindle. In such cases, anaphase II resulted in the production of a variable number of chromosomal aggregations that developed into pronuclei (3 to 6). In contrast, zygotes treated with CB during meiosis II developed spindles characteristic of untreated specimens; however, polar body formation was blocked, resulting in the development of usually two maternal pronuclei. Morphogenesis of *C. gigas* fertilized eggs is discussed in reference to similar processes in other species and with respect to strategies of triploid embryo production using CB.

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Introduction

Considerable emphasis has been given to the genetic manipulation of mollusks, in particular oysters, and its potential contribution to the domestication of aquatic fauna (Allen, 1987; Beaumont and Fairbrother, 1991). Triploid mollusks have been induced using a variety of methods: chemical, pressure, and temperature. Depending on when applied, cytochalasin B (CB) may inhibit the production of the first, second, or both polar bodies (Longo, 1972). Consequently, maternally derived chromosomes, normally segregated from the zygote, are retained, resulting in various states of ploidy. Although the effects of CB in oyster zygotes have been followed by cytological methods and flow cytometry (Allen, 1983; Chaiton and Allen, 1985; Guo *et al.*, 1992a, b), important cellular and developmental biological questions remain

unanswered regarding centrosome activity, spindle organization, and distribution of chromosomes. To establish the efficacy of procedures for chromosome manipulation, detailed analyses of the normal sequence of events during and after fertilization are required. In particular, analysis of components involved with meiotic maturation and pronuclear development and association in oysters may provide a better understanding of cellular and developmental processes in general and new insights into methods for increasing the effectiveness of chromosome manipulation.

Although electron microscopic studies have demonstrated ultrastructural features of oyster gametes (Galtsoff and Philpott, 1960; Galtsoff, 1964; Daniels *et al.*, 1971; Gutierrez *et al.*, 1978; Alliegro and Wright, 1983), we are unaware of studies dealing with the fine structure of gamete interactions and fertilization in this organism. Furthermore, investigations dealing with cellular and developmental aspects of oysters are relatively limited and unfocused (see Longo, 1983). The availability and abundance of both the organism and its gametes, as well as the relative ease of obtaining and handling eggs and embryos, make oysters a potentially ideal system for cellular and developmental studies. However, clear, baseline data regarding the morphology and biochemistry of gametogenesis, fertilization, and early embryogenesis are for the most part lacking, as are defined conditions and parameters to optimize development in this organism.

In a series of studies examining artificially activated oyster eggs for genetic studies, we investigated the course of fertilization events in CB-treated and untreated *Crassostrea gigas* eggs; we used electron microscopy and immunofluorescent techniques for the observation of DNA and microtubular organizations. Our observations bear on the structure and function of the meiotic spindle at first and second meiosis, germinal vesicle breakdown, and pronuclear development and association. These new observations are directly related to mechanisms whereby CB affects polar body formation and embryo ploidy.

Material and Methods

Mature oysters were obtained during the months from May to July from commercial producers in the vicinity of Bodega Bay, CA, and maintained in aquaria with running seawater at the Bodega Marine Laboratory, Bodega Bay, CA. The animals were stripped of their gametes: eggs were suspended in seawater and sperm were kept "dry" at 4°C until used for fertilization. Eggs were filtered through a 60-μm Nytex screen to remove gonadal tissue and debris, washed several times in seawater, and brought to a concentration of approximately 2×10^5/ml. The amount of sperm added to an egg suspension was determined for each experiment to avoid polyspermy. Generally, 20 μl of sperm from stripped testis was diluted into 10 ml seawater, which was then added to a 200-ml egg suspension. Fertilization and early development were carried out at 22°C.

At periodic intervals (3 or 6 min) following the mixing of sperm and eggs, 10-ml samples were taken and fixed (1) for electron microscopy (3% glutaraldehyde, 3% paraformaldehyde, 1% acrolein in seawater at 4°C; Longo and Anderson, 1970); (2) for 7-AAD (Molecular Probes, Eugene, OR) staining of DNA (3% paraformaldehyde and 0.1% glutaraldehyde in seawater at 4°C); and (3) for tubulin staining (50% methanol in 50 m*M* EGTA, pH 6.8 at −20°C). Preparations for electron microscopy were washed overnight in seawater (two changes), incubated in 0.5% OsO_4 in seawater for 1 h at 0°C, dehydrated in ethanol, and embedded in Spurr's embedding medium. Eggs were thin sectioned, stained with lead citrate and uranyl acetate, and viewed with a Hitachi 7000 EM electron microscope or thick sectioned (0.5 to 1.0 μm), stained with toluidine blue, and viewed with brightfield optics.

Specimens for DNA staining were washed overnight in seawater, incubated in a 3:1 solution of ethanol and acetic acid, and washed twice in phosphate buffered saline (PBS). Samples were stored at 4°C in PBS containing 0.02% NaN_3, reacted with 4 mg/ml $NaBH_4$ in PBS for 15 min, and stained in 1.5 μg/ml 7-AAD in PBS for 10 min. Stained preparations were washed two times in PBS, mounted in glycerol, and viewed with a Nikon inverted microscope fitted for epifluorescence. Specimens were also stained with the DNA intercalating dyes Hoechst 33342 and DAPI as described for 7-AAD. Fluorescent preparations were photographed using Kodak T-max 400 film.

Specimens prepared in cold methanol were washed two times in PBS and incubated in 1% BSA in PBS for 1 h, followed by a 1 h-incubation in anti-β-tubulin antibody (Calbiochem, San Diego, CA). Specimens were washed twice in PBS, incubated in FITC-goat antimouse antibody (1/20 dilution) for 1 h, washed in PBS, and mounted and viewed as previously described for 7-AAD specimens.

CB (stock of 1 mg/ml DMSO) was added to egg suspensions to a final concentration of 1 μg/ml just prior to insemination, immediately following first polar body formation or during the period (15 min) in which the first polar body is produced in controls. In the latter instance, after exposure to CB, zygotes were washed twice and resuspended in seawater. Controls consisted of fertilized eggs suspended in DMSO at a final concentration of 1 μl/ml. Samples of CB-treated eggs were fixed and processed as described above for untreated specimens.

The rates of sperm nuclear and female pronuclear enlargement were determined from specimens stained in 7-AAD. Video images of stained maternal and paternal genomes (about 50/time point) were projected onto a video monitor and their area determined. Specimens (50-60)

stained with antitubulin antibody during polar body formation were projected onto a video monitor and the length and width of the first and second meiotic spindles were measured. Results for nuclear and spindle measurements are presented as means ± SD.

Results

Morphogenesis of untreated eggs and zygotes

Structure of the unfertilized egg and germinal vesicle breakdown. The overall morphology of unfertilized eggs after incubation in seawater for 30 to 60 min took two forms: (1) Eggs were highly crenulated, pear-shaped, or both and possessed an intact germinal vesicle. (2) Eggs were essentially spheroidal (about 56 μm in diameter) and had germinal vesicles or were at metaphase I of meiosis. In virtually all preparations obtained from stripped ovaries, both germinal vesicle and metaphase I containing oocytes were present in approximately equal numbers. A small percentage (10 to 15%) appeared to be intermediate—*i.e.*, at a stage of germinal vesicle breakdown. Incubation in seawater for longer periods increased the number of eggs at metaphase I, but rarely did it approach 80% after 3-h incubation. Less than 1% of the eggs artificially activated spontaneously and developed beyond metaphase I.

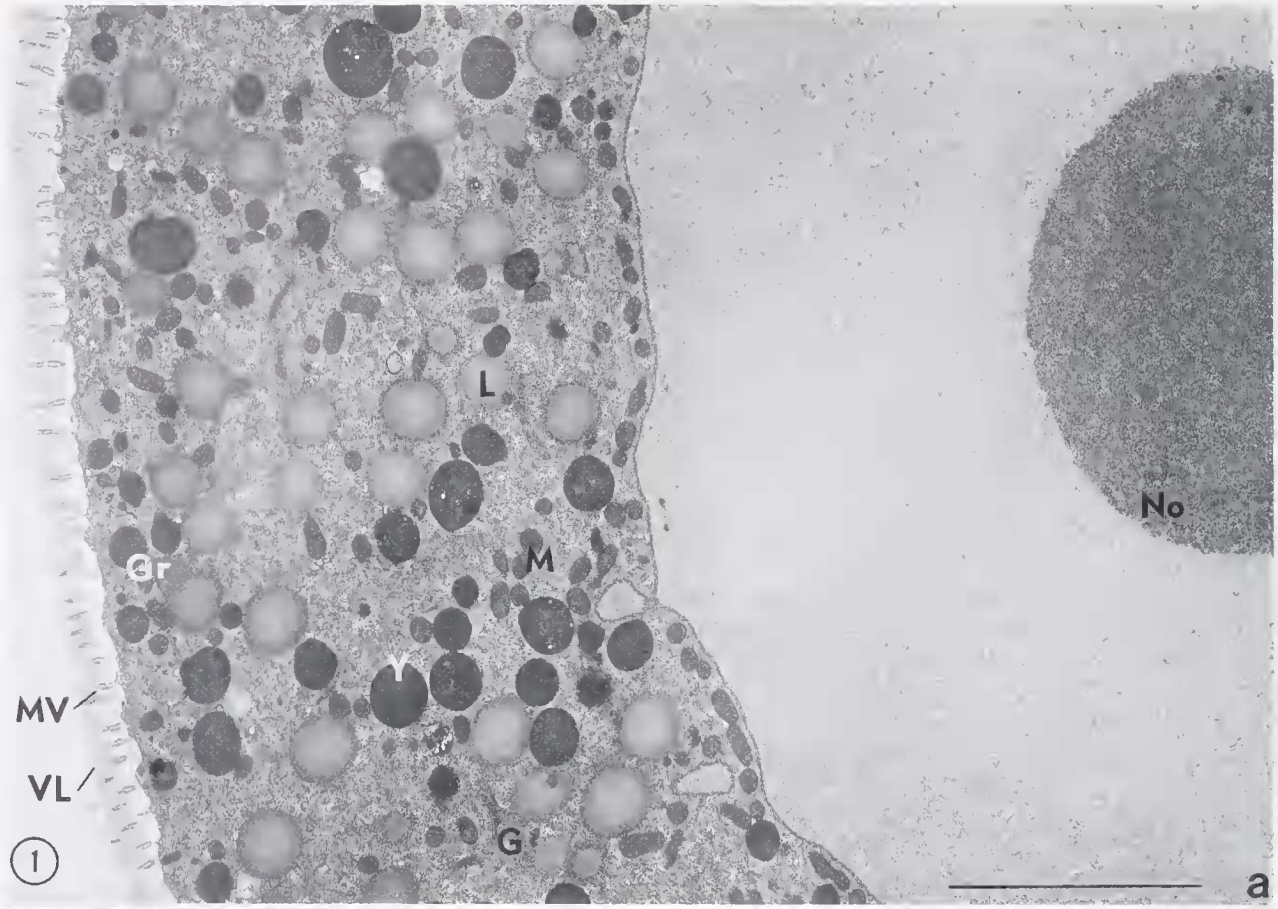
The germinal vesicle was spherical (about 30 μm in diameter) and possessed a relatively smooth surface (Fig. 1a, b). Tetrad chromosomes were distinguished in specimens stained with 7-AAD but not in DAPI- and Hoechst-stained specimens because of high background staining (Fig. 1b). A single, large nucleolus was a distinct feature of the germinal vesicle (Fig. 1a).

The cytoplasm of eggs having germinal vesicles contained two relatively small, stellate foci of antitubulin staining that were usually positioned at one pole of the germinal vesicle (Fig. 1c). Mitochondria, cisternae of endoplasmic reticulum, Golgi and yolk bodies were distributed apparently at random throughout the egg cytoplasm (Figs. 1a, 2). The endoplasmic reticulum was present as relatively small cisternae that did not form large aggregations or extensive networks. Granules, posessing a homogeneous electron-dense texture, were observed along the egg's periphery (Figs. 1a, 2). The egg surface was reflected into prominent microvilli, about 1 μm in length by 0.1 μm in diameter (Fig. 2), that projected through a vitelline layer composed of a fine filamentous material. Along the external surface of the vitelline layer was a lamina, perhaps adherent jelly, that was suspended from the tips of the microvilli (Figs. 2, 3).

Germinal vesicle breakdown was initiated spontaneously or apparently as a result of sperm-egg interaction. In the latter case, sperm, having undergone the acrosome reaction, were observed at the tips of microvilli (Fig. 3). Other than the presence of sperm, germinal vesicle breakdown in fertilized and unfertilized eggs appeared to be morphologically comparable (Fig. 4). There was little, if any, apparent modification in the structure of the nucleolus (Fig. 5). In fact, an intact nucleolus persisted throughout fertilization (see below). The tetrad chromosomes progressively condensed, and this was accompanied by a crenulation of the surface of the germinal vesicle (Figs. 4, 5a; inset 5c). Nuclear pores disappeared and fenestrations developed within the nuclear envelope such that eventually the condensing chromosomes were loosely surrounded by cisternae derived from the nuclear envelope (Fig. 5b, c). Concomitantly, aggregations and anastomosing networks of endoplasmic reticulum became prominent throughout the cytoplasm (Fig. 5c, d; inset 5d).

As the chromosomes condensed, they progressively aggregated onto a plate-like mass in the center of the egg (Fig. 4c). Concomitant with this aggregation, the two stellate foci of antitubulin staining—formerly positioned at one pole of the germinal vesicle—enlarged, became closely associated with the condensing chromosomes, and eventually localized to poles of the first meiotic spindle. The first meiotic spindle then moved peripherally to become positioned within the cortex (Fig. 4e, f). The size and orientation of the first meiotic spindle are particularly noteworthy in contrast to the second (Fig. 4e, f). The long axis of the first meiotic spindle was 12.4 ± 1.0 μm and its diameter at the metaphase plate measured 5.4 ± 0.55 μm. In all specimens examined, the long axis of the spindle was positioned normal to the egg surface.

Polar body formation. In those eggs inseminated at metaphase I or the germinal vesicle stage, gamete interaction triggered the continuation of meiotic maturation. At anaphase I, diads disjoined; the most cortically positioned of the two groups of chromosomes became enclosed within the first polar body (Figs. 4g, h; 6). Concomitantly, that portion of the spindle remaining within the zygote regressed. This was followed by the reorganization of the diad chromosomes onto the metaphase plate of the second meiotic spindle (Fig. 6a), which was distinctly different from the first. (1) The size of the second meiotic spindle, both its long axis (7.4 ± 0.6 μm) and its diameter (2.9 ± 0.29 μm), were approximately one half that of the first. (2) Its orientation when formed was exactly opposite that of the first; *i.e.*, its long axis was positioned parallel to the egg's surface (Fig. 6b). Just prior to anaphase II, the second meiotic spindle rotated so that its long axis became perpendicular to the egg surface (Fig. 6c, d). Accompanying anaphase II was the formation of the second polar body, which became positioned subjacent or lateral to the first (Figs. 6e, 7). The maternal chromosomes remaining in the zygote swelled and became invested by a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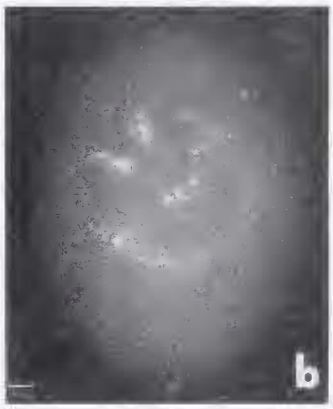
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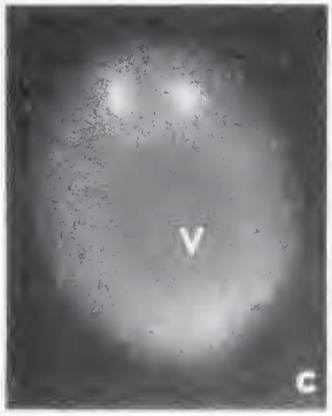
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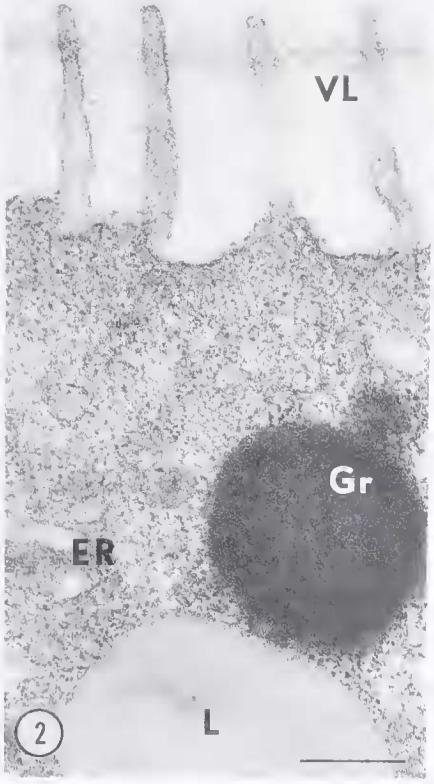
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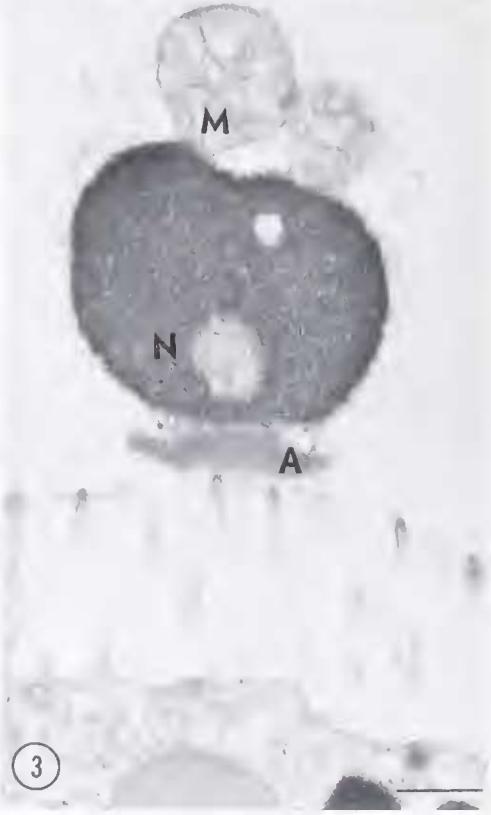
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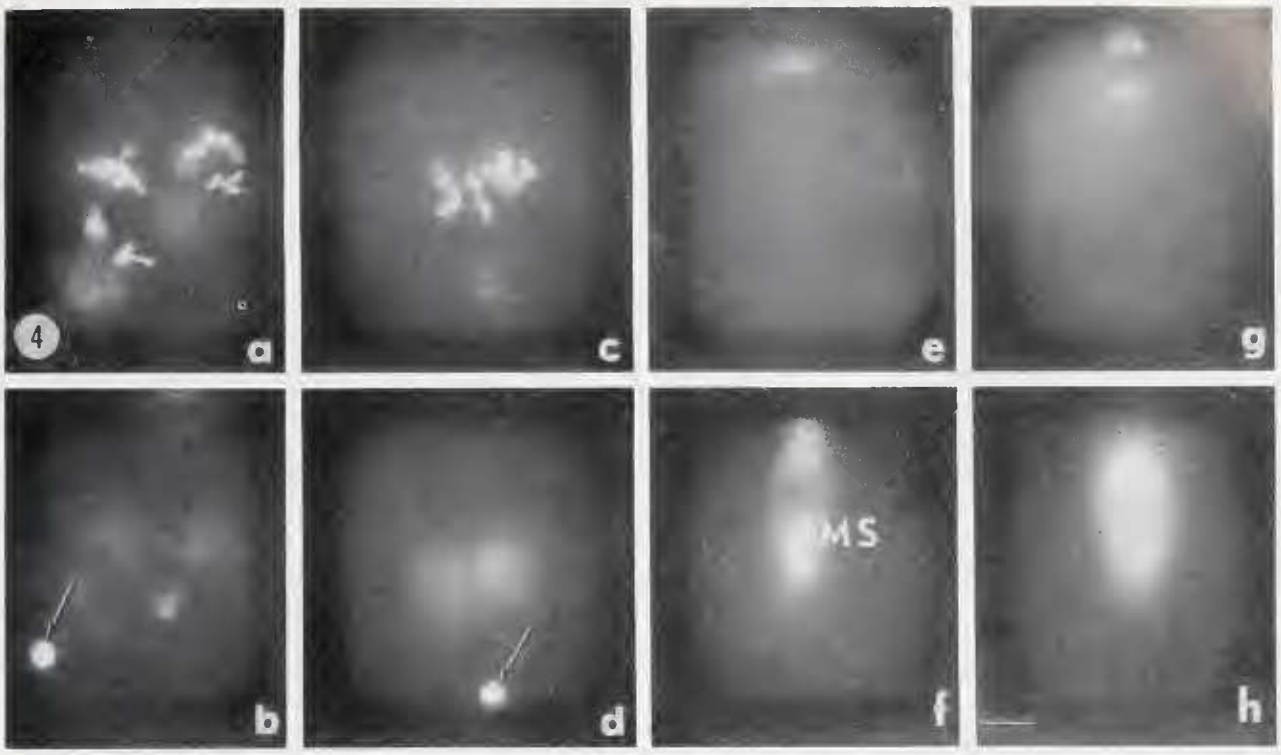


Figure 4. Condensation of tetrads and their localization within the egg during metaphase and anaphase I. Progressive stages in the condensation and alignment of tetrads onto the metaphase plate takes place in the midregion of the egg (a, c); the metaphase spindle (MS) then moves to and becomes positioned at one pole of the egg with its long axis perpendicular to the egg's surface (e, f). (b, d) Micrographs of fertilized eggs shown in Figure 4a and c, respectively, at levels to demonstrate incorporated sperm nuclei (arrows). (g, h) Specimen at anaphase I depicting segregating chromosomes (g) and the meiotic spindle (h). (a–e, g) Preparations viewed to show 7-AAD-stained parental genomes. (f, h) Antitubulin-stained meiotic spindles. Scale bar = 10 μm.

nuclear envelope to form the female pronucleus (Figs. 6g, h; 7, 11).

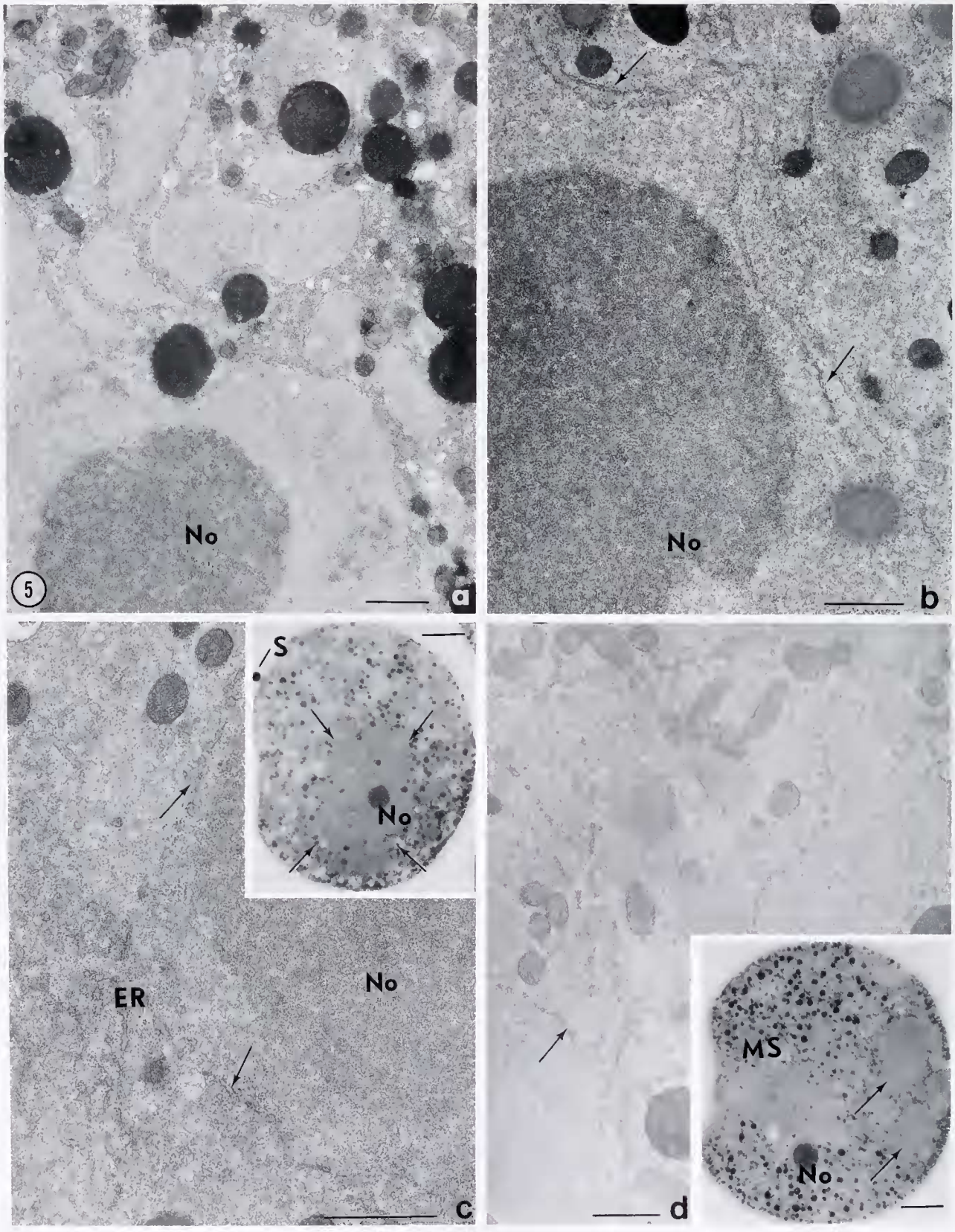
In conjunction with the spindle pole remaining in the egg, a system of microtubules assembled and enveloped the developing female pronucleus (Fig. 6 f–g). Accompanying these changes, a sperm aster developed in association with the transforming sperm nucleus (Fig. 6f, g). With the continued growth of the sperm aster, the system of microtubules associated with the female pronucleus became smaller and eventually disappeared.

Half-spindles taken into the first and second polar bodies quickly regressed following the formation of each polar body and appeared as fluorescent streaks (Fig. 6b–d). Chromosomes of the first polar body usually failed to dis-

Figure 1. Unfertilized oyster eggs containing germinal vesicles. (a) The surface of the germinal vesicle is relatively smooth and devoid of extensive folds. The cytoplasm consists of a fairly homogeneous distribution of yolk (Y), lipid droplets (L), Golgi (G), and mitochondria (M). Although cisternae of endoplasmic reticulum are distributed throughout the egg cytoplasm, they do not form large aggregations as seen in eggs in which the germinal vesicle has broken down (see Fig. 5). Granules (Gr), smaller and with a more homogeneous content than yolk bodies, occupy the cortical region of the egg. (b, c) Fluorescence micrographs of 7-AAD- and antitubulin-stained eggs demonstrating tetrad chromosomes (b) and foci of microtubules (c) at one pole of the germinal vesicle (V). MV, microvilli; VL, vitelline layer; No, nucleolus. Scale bar = 5 μm.

Figure 2. Cortex of an unfertilized egg depicting microvilli, granules (Gr), lipid droplets (L), elements of the endoplasmic reticulum (ER), and vitelline layer (VL). Structural changes in the egg cortex were not apparent as a result of insemination. Scale bar = 0.5 μm.

Figure 3. Sperm-egg interaction. The sperm has undergone the acrosome reaction and is in contact with the egg's microvilli. N, sperm nucleus; M, sperm mitochondria; A, basal portion of the sperm acrosome. Scale bar = 0.5 μm.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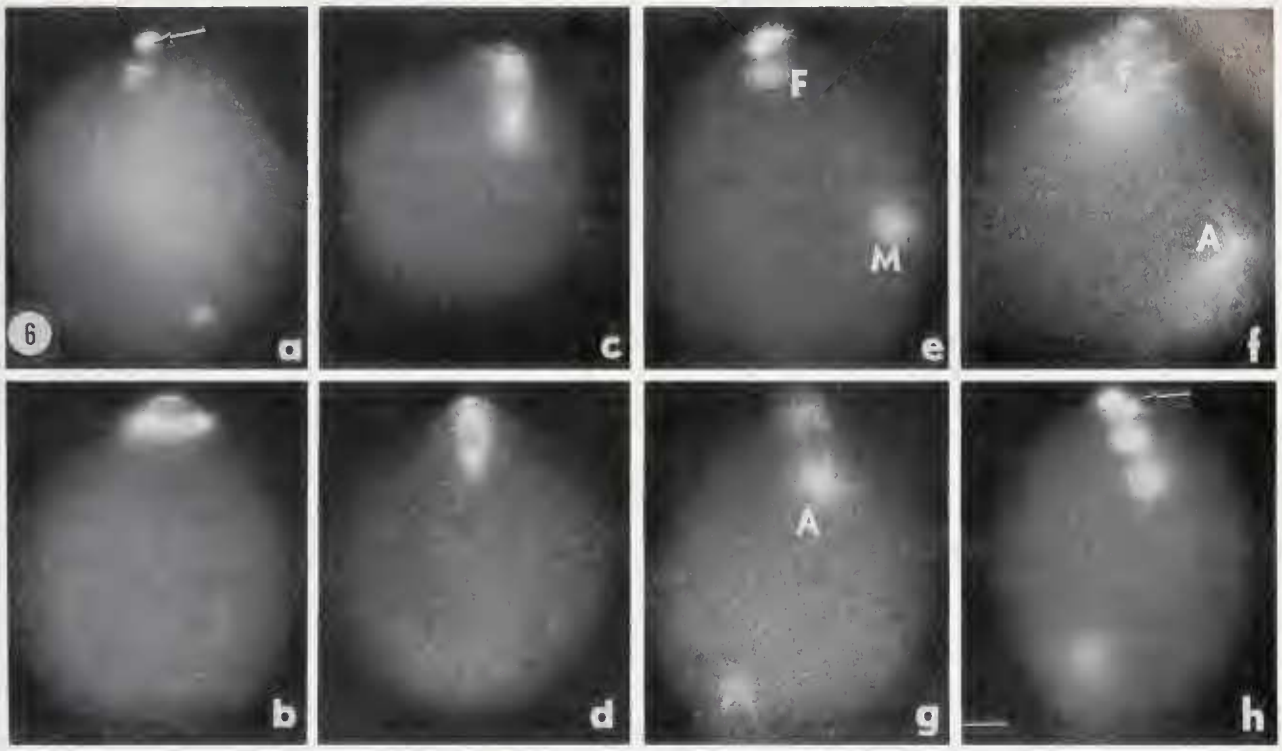

Figure 6. Polar body formation. (a–b) Fertilized egg shortly following the formation of the first polar body (arrow). The chromosomes remaining in the egg following anaphase I become organized on the second meiotic spindle, which is significantly smaller than the first and is initially oriented tangential to the egg's surface (b). (c, d) The second meiotic spindle rotates and becomes positioned normal to the egg's surface (c) and undergoes anaphase II (d). (e, f) Fertilized eggs following anaphase II. (e) The maternal (F) and sperm (M) chromatin enlarge to form female and male pronuclei, respectively. (f, g) Microtubule arrays develop in conjunction with the female and male (sperm aster) pronuclei. (g) The microtubule array associated with the female pronucleus (F) eventually regresses with the continued development of the sperm aster (A). (h) Zygote depicting apposed male and female pronuclei and two polar bodies (arrow). Preparations a, e, h are viewed to show 7-AAD staining and the maternal and paternal chromatin; specimens b, c, d, f, g viewed to show antitubulin-stained structures. Scale bars = 10 μm.

perse and were not delimited by a nuclear envelope (Fig. 7). In contrast, chromosomes of the second polar body dispersed and were surrounded by a nuclear envelope (Fig. 7).

Sperm incorporation and male pronuclear development. Sperm-egg interaction leads to gamete membrane fusion and, as a consequence, the incorporation of the sperm nucleus, mitochondria, and axonemal complex (Figs. 8a–d). The projection of egg cytoplasm that formed at the site of sperm entry—the fertilization cone—was relatively small, usually just slightly larger than the sperm head. The pore connecting the fertilization cone with the egg cortex widened, thereby allowing the contents of the spermatozoan to move into the cortex (Fig. 8a, b). This was followed by sperm chromatin dispersion; an apparently rapid process in which the condensed, granular chromatin

Figure 5. Germinal vesicle breakdown is accompanied by the formation of extensive convolutions of the nuclear envelope (a) and aggregations of endoplasmic reticulum (ER). (b, c) The nuclear envelope loses its pores and is severed at multiple sites to form cisternae (arrows) that are morphologically similar to endoplasmic reticulum. (d and inset) Elongate networks and aggregations of endoplasmic reticulum (arrows) that form in eggs having undergone germinal vesicle breakdown. Aggregations of endoplasmic reticulum are sufficiently large that they are readily discerned as "clear" staining areas in light microscopic preparations (inset d; arrows). A prominent feature of germinal vesicle breakdown in oyster eggs is the retention of an intact nucleolus (No; a–c, insets c and d). Inset c: Stained thick section showing a sperm prior to its incorporation into the egg cortex and an intact nucleolus (No) in the area of the disrupted germinal vesicle (arrows). MS, meiotic spindle. Scale bar = 1 μm (Fig. 5a–d); 10 μm (insets).

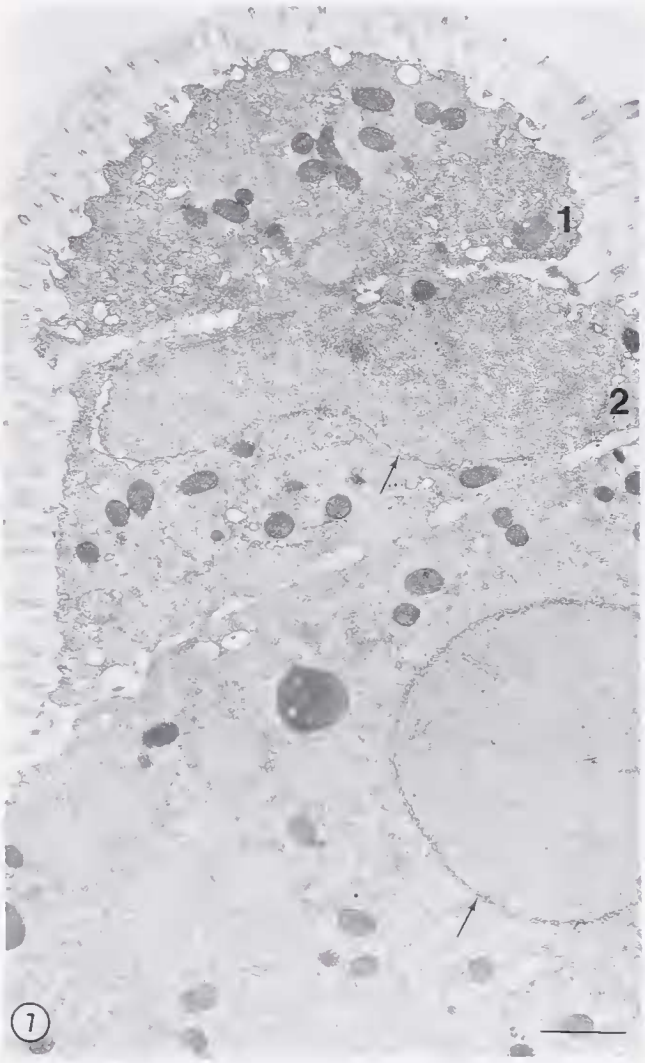

Figure 7. First and second polar bodies and female pronucleus of a zygote following meiotic maturation. Chromatin within the second polar body (2) and zygote becomes organized within a nuclear envelope (arrows). Chromatin in the first polar body (1) usually remains devoid of delimiting membranes. Scale bar = 1 μm.

became dispersed into an aggregation of fine chromatin filaments (Fig. 8c, d). Analysis of sperm chromatin dispersion revealed that it consisted of basically two phases of enlargement: (1) a slow rate that occurred during germinal vesicle breakdown and polar body formation, and (2) a much faster rate of expansion corresponding with female pronuclear enlargement (Fig. 9). The sperm chromatin remained in a dispersed state, free of a nuclear envelope throughout the period of polar body formation. Concomitant with the formation of the female pronucleus, vesicles and elongate cisternae aggregated along the surface of the dispersed sperm chromatin. These elements fused together and acquired pores to constitute the nuclear envelope (Figs. 10, 11).

Pronuclear apposition and formation of the first cleavage spindle. After formation of the pronuclei, the male pronucleus migrated to the female pronucleus, which was either centrally placed within the zygote or situated just subjacent to the first and second polar bodies (Fig. 11). Within the two pronuclei were dense accumulations, structurally reminiscent of the nucleolus derived from the germinal vesicle (Fig. 11c). These structures appeared within the pronuclei with no apparent change in the nucleoli derived from germinal vesicles (Fig. 11b, insets b, c).

With the apposition of the pronuclei, two antitubulin-staining foci appeared; these became the poles of the mitotic spindle (Fig. 12a–c). Simultaneously, the male and female pronuclei underwent prophase; *i.e.*, their chromosomes condensed (Fig. 12) and their nuclear envelopes broke down (Fig. 12e–h). Subsequently, the maternally and paternally derived chromosomes became positioned on the metaphase plate of the mitotic spindle assembled for first cleavage (Fig. 12h). A similar pattern of morphogenesis was also observed in polyspermic eggs. The male pronuclei, each of which possessed a sperm aster (Fig. 12d; see also CB Effects), migrated into association with one another and the female pronucleus. A multipolar spindle formed from this association; the number of poles it possessed was a direct reflection of the extent of polyspermy.

CB effects

Continuous exposure to CB. Many of the processes occurring in zygotes treated with CB were similar to, if not the same as these in the controls; therefore, only those processes directly affected by CB are described here. In the continuous presence of CB, formation of both polar bodies was inhibited. Anaphase I occurred and the projection of cytoplasm normally budded from the egg to form a polar body developed; however, it was not severed from the zygote (Fig. 13a–d). Consequently, all the diad chromosomes were retained within the zygote (Fig. 13c). The spindle regressed and all the chromosomes became reorganized on a tripolar spindle formed within the egg cortex (Fig. 13e–f). The spindle had the shape of an equilateral triangle in which the length of each side was approximately equal to the long axis of spindles that formed at metaphase II in untreated eggs. Interestingly, the tripolar spindle was oriented within the cortex so that a base or an apex was positioned directly subjacent to the cell surface. Chromosome separation followed and a variable number (3–6) of female pronuclei were eventually assembled, most likely as a result of chromosome segregation and aggregation at anaphase II and pronuclear assembly, respectively (Longo, 1972).

CB application during first polar body formation. When CB was administered only during the period of first polar

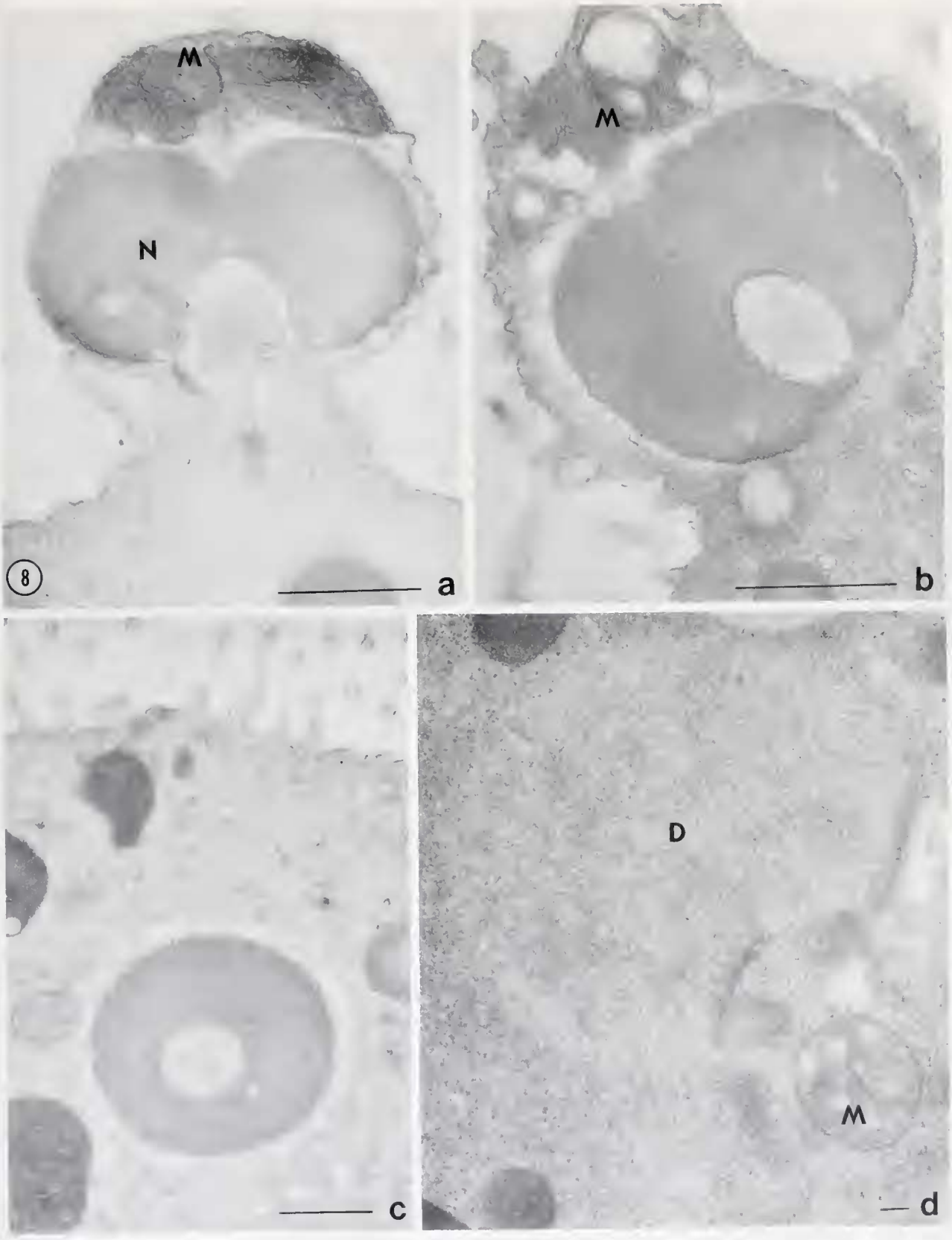

Figure 8. (a–d) Sperm incorporation. (a, b) A small fertilization cone, essentially encompassing the sperm nucleus (N) and mitochondria (M), forms following gamete membrane fusion. (c, d) Both the sperm nucleus and mitochondria move through this projection of cytoplasm into the egg cortex where the condensed chromatin disperses (c) into an aggregation of fine filaments (d). D, dispersed sperm chromatin. Scale bars = 1 μm (Fig. 8a–c); 0.1 μm (Fig. 8d).

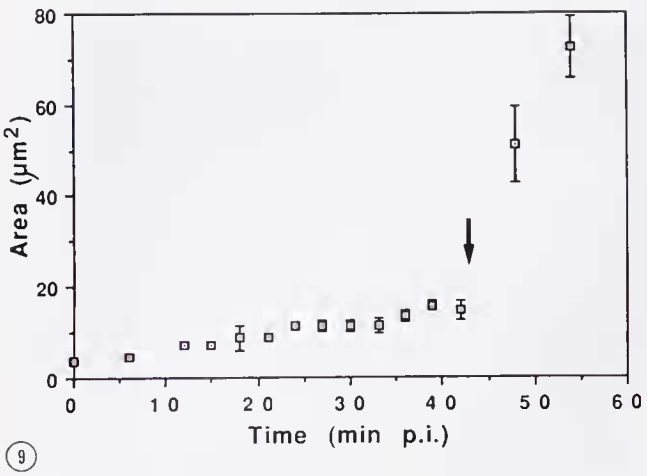

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Figure 9. Sperm nuclear enlargement consists of at least two stages: a phase consisting of a relatively slow rate of expansion that occurs during germinal vesicle breakdown and polar body formation [0 to ~40 min postinsemination (p.i.)]. This is followed by a phase of rapid expansion that occurs in conjunction with enlargement of the female pronucleus. The arrow indicates the time meiotic maturation is concluded and development of a maternal pronucleus is initiated.

body formation (15 min), chromosomes normally extruded from the zygote in the first polar body were retained. As in the case of zygotes continuously exposed to CB, all of the maternal chromosomes became positioned on a tripolar spindle (Fig. 14a, b). When CB-treated eggs were washed and resuspended in fresh seawater following the period of first polar formation (20 min after insemination), a polar body did not always form. When it did, it was morphologically similar to the second polar body of control zygotes (Fig. 15a); *i.e.*, it possessed dispersed chromatin surrounded by a nuclear envelope (Fig. 15). In these cases a variable number (3–6) of female pronuclei formed in a manner described above.

CB application during second polar body formation. Zygotes treated with CB during formation of the second polar body possessed a first polar body and had their chromosomes organized on second meiotic spindles typical of controls (Fig. 16a, b). In the presence of CB, the spindle rotated so that its long axis became positioned perpendicular to the egg surface (Fig. 16c, d). Anaphase II ensued and a projection of cytoplasm encompassing chromosomes normally expelled with the polar body formed (Fig. 16e). However, the cytoplasm was not severed from the zygote and all of the chromosomes of the second meiotic division were retained (Fig. 16e). Three pronuclei formed within such zygotes, two of maternal origin and one derived from the sperm (Fig. 16f); all underwent prophase as in controls. The condensed chromosomes of all three pronuclei became organized on a single mitotic spindle (Fig. 16g, h).

Discussion

The observations presented here document changes in fertilized eggs of the Pacific oyster, *Crassostrea gigas*. Meiotic maturation, assembly of the male and female pronuclei, pronuclear association, and subsequent development of the first cleavage spindle in CB-treated and untreated specimens have been observed with both light and electron microscopy.

Egg structure at fertilization

Essentially two morphological forms of *C. gigas* eggs were present from stripped, ovarian preparations. This variation in overall structure and in meiotic maturation has been noted previously and may be related to the method of gamete acquisition (Longo, 1983). The significance of these two forms with respect to gamete maturation, fertilizability, and potential for development is unclear. Numerous studies (Longwell and Stiles, 1968; Downing and Allen, 1987; Stephano and Gould, 1988; Scarpa and Allen, 1992) have demonstrated that oyster eggs, obtained by stripping, can be fertilized and are capable of embryogenesis. Observations presented here indicate that with the exception of germinal vesicle breakdown and first meiotic spindle assembly, both forms undergo virtually identical processes at fertilization.

It is noteworthy that reports of the timing of various stages of fertilization vary considerably (Galtsoff, 1964; Longwell and Stiles, 1968; Stiles and Longwell, 1973; Stanley *et al.*, 1984; Downing and Allen, 1987). Although parameters for optimal fertilization and early embryogenesis have not been well-defined, temperature and salinity as well as the mode of gamete acquisition no doubt have profound effects on the timing of fertilization events and subsequent development (see Scarpa and Allen, 1992). The use of serotonin to induce germinal vesicle breakdown (Osanai, 1985) provides a means of obtaining homogeneous populations of eggs so that more synchronous development can be achieved. With respect to events reported here, events in serotonin-treated eggs are essentially the same morphologically as in eggs obtained by stripping (Stephano and Gould, 1988). The timing of fertilization events, however, is related to the meiotic stage of the egg at insemination (Scarpa and Allen, 1992).

Egg structure and the events of *C. gigas* fertilization, involving germinal vesicle breakdown and the formation of the first meiotic spindle, are similar to structures and processes reported for other mollusk eggs (Longo and Anderson, 1969, 1970; Longo, 1983; Komaru *et al.*, 1990; Longo and Scarpa, 1991; see also Dessev and Goldman, 1988; Dessev *et al.*, 1989; Stricker and Schatten, 1989). Notable differences, however, were observed in the present study. Retention of the nucleolus is a consistent feature of *C. gigas* eggs that undergo germinal vesicle breakdown.

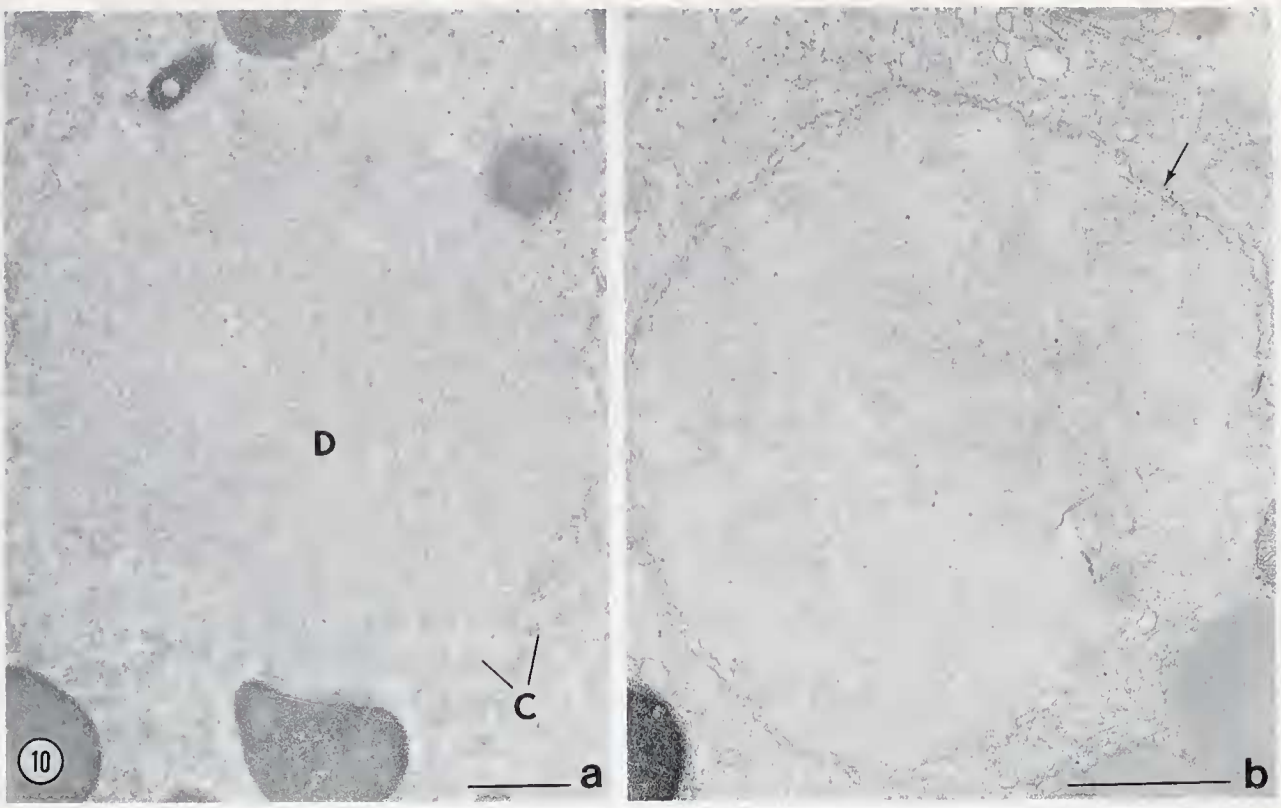


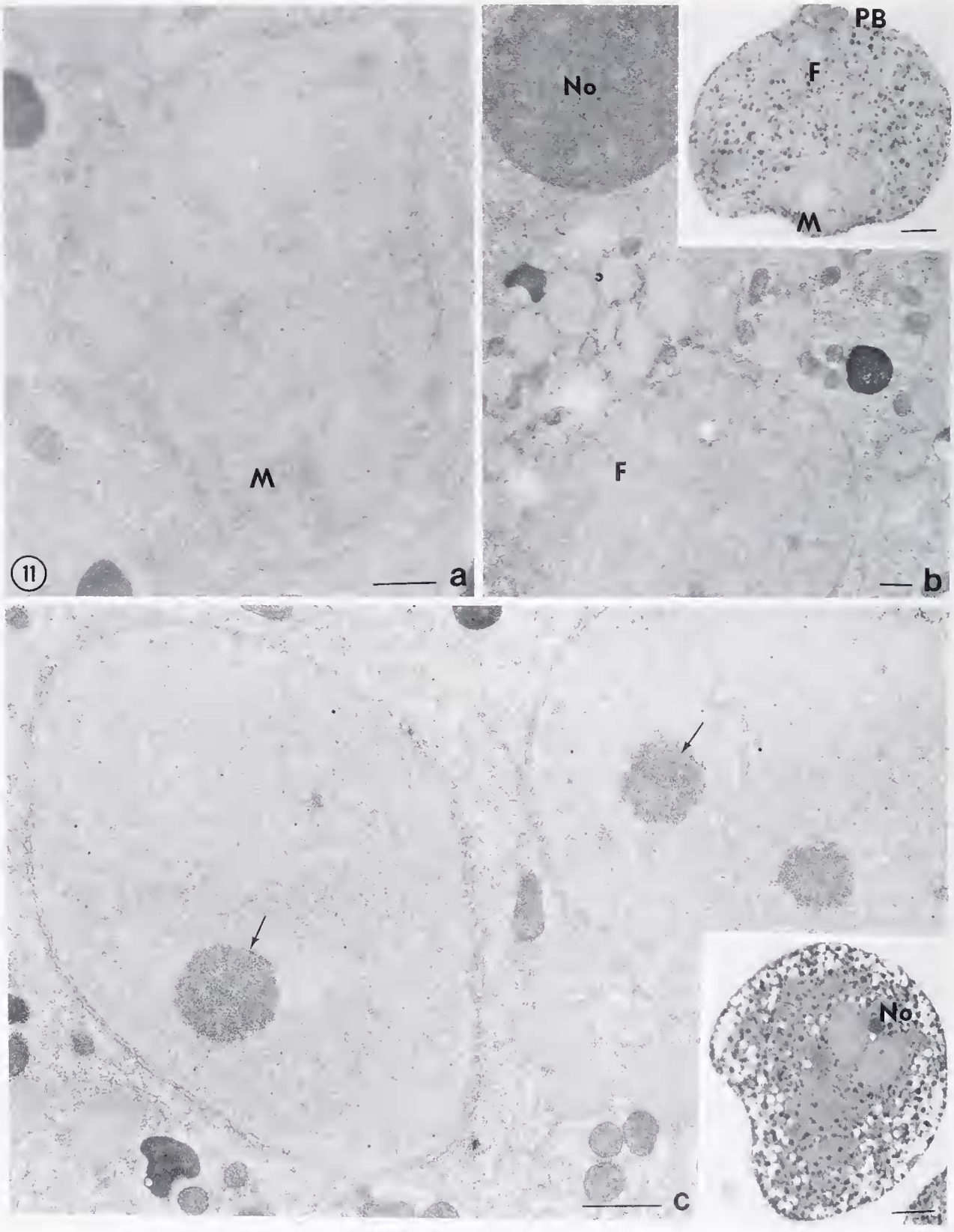
Figure 10. (a, b) Formation of the male pronuclear envelope. (a) Following polar body formation, cisternae (C) surround the dispersed sperm chromatin (D) and (b) fuse to form a nuclear envelope (arrow). Scale bar = 1 μm.

This structure did not appear to diminish appreciably in size throughout the period of fertilization (up to 1 h). In mitotic cells, loss of the nucleolus is due to the cessation of rRNA synthesis, coupled with the continued processing of ribosomal precursors (Anastassova-Kristeva, 1977; Hadjiolov, 1985). Failure of these events presumably would lead to the retention of a nucleolus as seen here.

Major events in the breakdown of the germinal vesicle are the loss of nuclear pore complexes and the vesiculation of the nuclear envelope into cisternae that are structurally reminiscent of elements of the endoplasmic reticulum. Concomitant with the breakdown of the nuclear envelope is the formation of networks and aggregations of endoplasmic reticulum. Although the surface area of the nuclear envelope measures about 2900 μm^2, it is doubtful that all membranous elements comprising the networks and aggregations that appear with germinal vesicle breakdown are, in fact, derived from the nuclear envelope. This impression is based on preliminary observations (F. J. Longo, pers. obs.) indicating that the number and size of endoplasmic reticulum aggregations comprise a greater area than can be accommodated by the nuclear envelope of the germinal vesicle. Hence, we suggest that concomitant with germinal vesicle breakdown, cisternae of endoplasmic reticulum throughout the egg are organized into dense aggregations and networks. Cisternae derived from the breakdown of the nuclear envelope may be a part of these membranous arrays.

Alterations in the endoplasmic reticulum seen in *C. gigas* eggs/zygotes are similar to those described for starfish oocytes (Kato *et al.*, 1990) and reminiscent of dynamic changes that occur in the endoplasmic reticulum of sea urchin eggs at fertilization, presumably in relation to the initiation of egg activation (Terasaki and Jaffe, 1991; see also Campanella *et al.*, 1984; Speksnijder *et al.*, 1993). Morphogenic changes of the endoplasmic reticulum may play a similar role in the regulation of fertilization processes in *C. gigas* eggs.

Changes in the surface structure of the egg, *i.e.*, crenated to smooth, appeared to be coupled to nuclear events and are largely inhibited by CB (F. J. Longo, pers. obs.). The cortex of *C. gigas* eggs was similar to that of other spiralian eggs (Rebhun, 1962; Fallon and Austin, 1967; Longo and Anderson, 1969, 1970; Hylander and Summers, 1977). Previous studies (Galtsoff, 1964; Osanai, 1969) refer to either a cortical granule reaction or fertilization envelope formation in *Crassostrea;* such processes were not apparent, nor were changes in microvillar structure and the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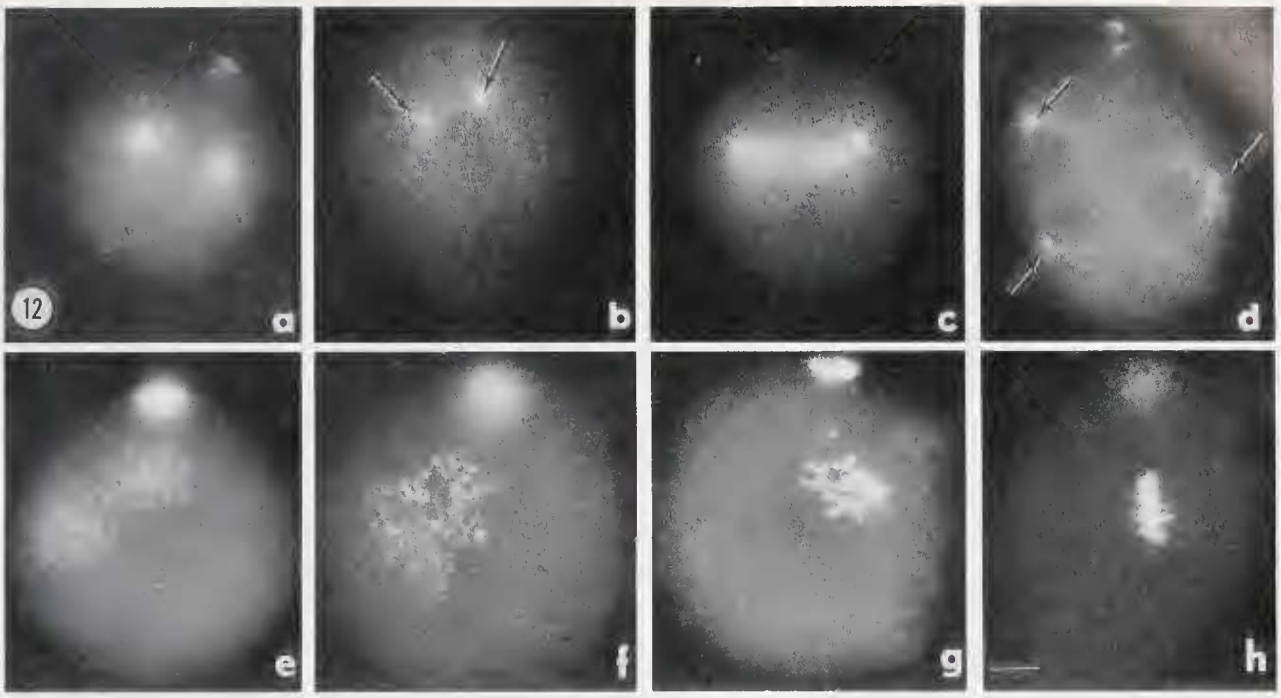

Figure 12. Antitubulin- and 7-AAD-stained specimens depicting the development of microtubule arrays (a–d) and the morphogenesis of the maternally and paternally derived chromatin following pronuclear apposition (e–h). (a–c) Stages in the development of the mitotic spindle; the developing spindle poles are shown at the arrows. (d) Polyspermic egg in which each male pronucleus is associated with a developing sperm aster (arrows). (e, f) Apposed male and female pronuclei undergoing chromatin condensation. (g, h) Coalescence (g) and alignment (h) of the paternally and maternally derived chromosomes on the metaphase plate of the first cleavage spindle. P, polar bodies. Scale bar = 10 μm.

vitelline layer obvious with the methods employed here (see also Alliegro and Wright, 1983).

Centrosomes and first and second meiosis

Observations presented here indicate that germinal vesicle eggs of *C. gigas* possess two foci of radiating microtubules that may be intimately involved in meiotic spindle formation. At the time of germinal vesicle breakdown, they appear to establish the poles of the first meiotic apparatus. Such foci are reminiscent of those observed in starfish oocytes (Schroeder, 1985; Otto and Schroeder, 1984) and in other cell types (see Kalt and Schliwa, 1993) where they organize microtubules during both interphase and mitosis and are referred to as centrosomes.

The first meiotic spindle of *C. gigas* forms in the central portion of the egg and then moves to and becomes positioned perpendicularly to the egg cortex. A similar morphogenesis has been described for vertebrate and invertebrate eggs (Gard, 1992; see Longo, 1983). It is noteworthy that in the present case, migration to and orientation of the spindle within the egg cortex does not appear to be affected by CB. This is in contrast to studies with maturing mammalian oocytes indicating that microfilaments are involved in the centrifugal displacement of the first metaphase spindle (Longo and Chen, 1985; Maro *et al.*, 1986; Van Blerkom and Bell, 1986; Alexandre *et al.*, 1989; Ryabovra *et al.*, 1986).

The size and orientation of the first meiotic spindle of *C. gigas* are comparable to those previously reported and

Figure 11. (a–c) Male (M) and female (F) pronuclei prior to (a, b), and following their apposition (c). (b) The nucleolus (No) derived from the former germinal vesicle is situated near the female pronucleus (F). (c) Both the male and female pronuclei accumulate dense aggregations, reminiscent of nucleoli (arrows). Inset b: Male (M) and female (F) pronuclei prior to their migration. The area partially surrounding the male pronucleus and essentially devoid of granular organelles comprises a portion of the sperm aster. Inset c: Apposed male and female pronuclei adjacent to the nucleolus derived from the former germinal vesicle. PB, first and second polar bodies. Scale bar = 1 μm (Fig 11a–c); 10 μm (insets).

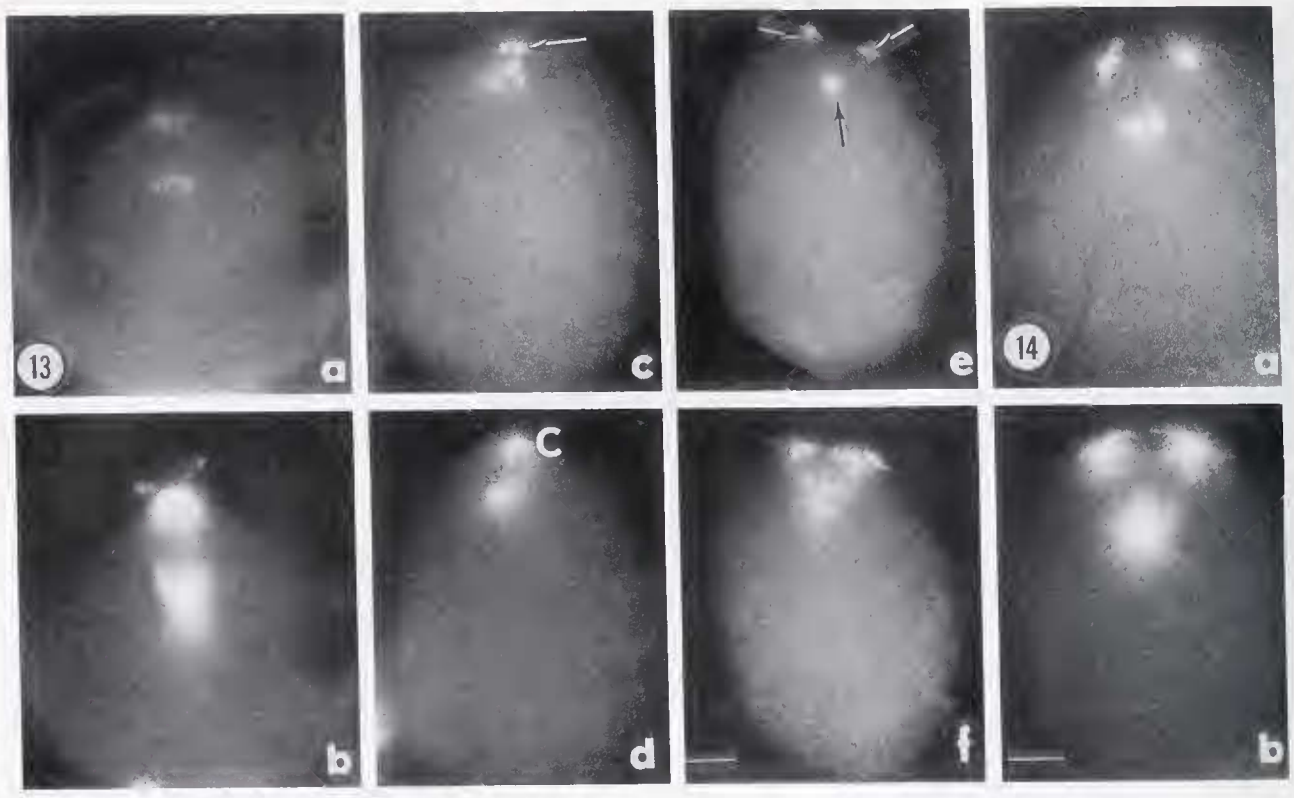

Figure 13. (a, c, e) 7-AAD- and antitubulin-stained (b, d, f) eggs treated continuously with cytochalasin B. (a–d) Because first polar body formation is inhibited, the chromosomes (arrows) and the spindle pole (C) normally expelled at meiosis I are retained within the zygote. (e, f) All of the chromosomes (arrows) then become part of a tripolar spindle (f). Scale bar = 10 μm.

Figure 14. Tripolar spindle that forms in fertilized eggs treated with cytochalasin B during meiosis I. Specimen stained with 7-AAD (a) and antitubulin (b). Scale bar = 10 μm.

for other mollusk species that have been examined (Inaba, 1936; Longo and Anderson, 1969, 1970; Kuriyama *et al.*, 1986; Kuraishi and Osanai, 1988). However, the second meiotic spindle differs from the first in both its size and initial orientation. The basis for differences in size and orientation of the second meiotic spindle is not clear. Meiotic spindles oriented parallel to the egg cortex are not uncommon, particularly in mammals (Longo, 1987; see also Gard, 1992). As far as we are aware, differences in spindle size of meiotic eggs are unusual, and how they might be regulated is not clear. An examination of previously published observations of fertilized Japanese oyster eggs does show what may be differences in size and orientation of the first and second meiotic spindles (Inaba, 1936; Kuraishi and Osanai, 1988). Whatever the scheme to explain such differences, it will have to take into account observations that the axes of the tripolar meiotic spindle formed in CB-treated eggs are approximately equal to the spindle length of the second meiotic apparatus. Furthermore, mechanisms underlying spindle orientation have not been established, although microtubules have been implicated in this process (Hyman, 1989; Kropf *et al.*, 1990; Fernandez *et al.*, 1990; Gard, 1992).

The array of microtubules that develops in conjunction with the forming female pronucleus disappears by pronuclear migration, suggesting that it may have little to do with this process. Concomitant with the increase and regression of the microtubular array associated with the female pronucleus is the development of the sperm aster, which accompanies the male pronucleus in its migration. The sperm aster remains in proximity with the associated pronuclei and with the onset of prophase appears to divide into two foci that form the poles of the cleavage spindle (Sluder *et al.*, 1985; Schatten *et al.*, 1986). This morphogenesis and the disappearance of microtubule arrays associated with the female pronucleus suggest that centrosomes of *C. gigas* embryos are paternally derived as described for other species (Longo and Anderson, 1969, 1970; Kuriyama *et al.*, 1986; Schatten *et al.*, 1986; Sluder *et al.*, 1985, 1989.) The presence of multiple sperm asters in polyspermic oyster eggs, their apparent contribution to the formation of multipolar cleavage spindles, and mi-

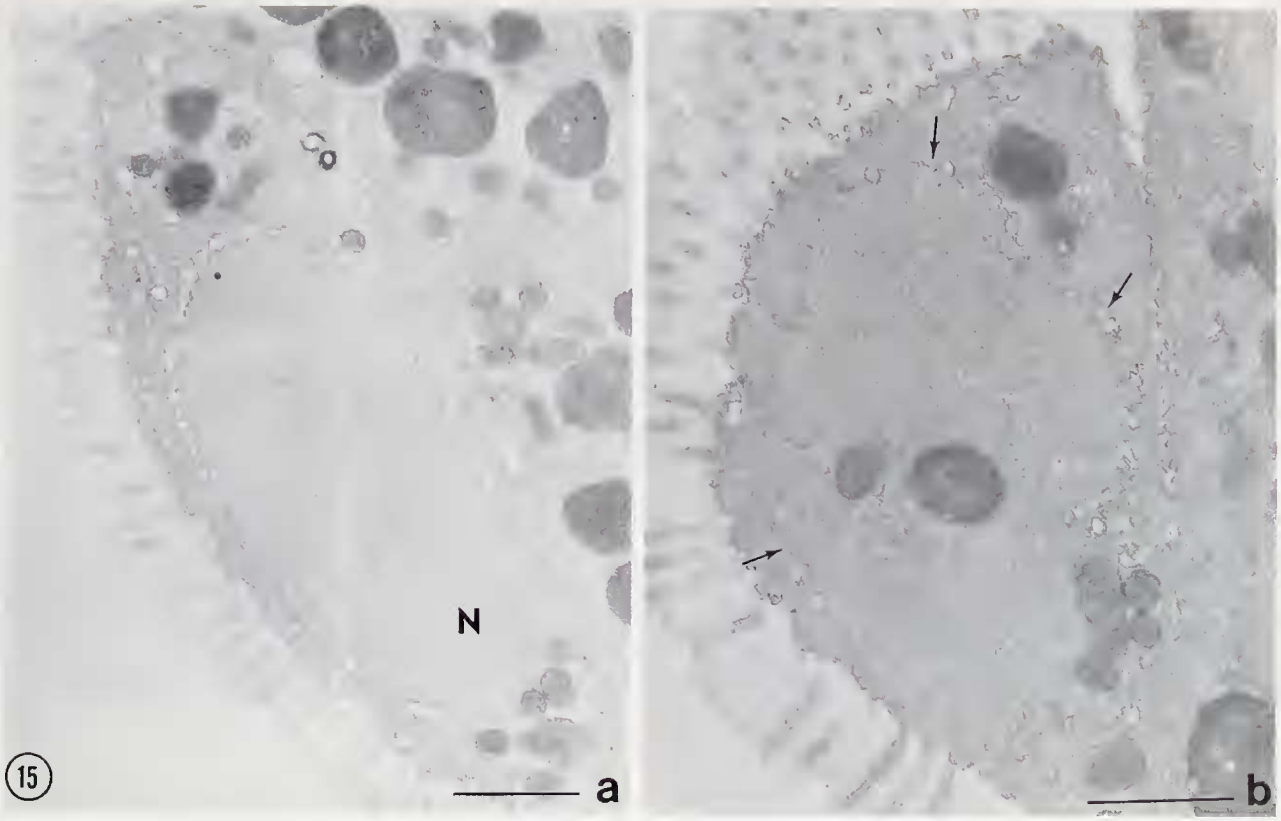

Figure 15. Polar bodies formed in eggs treated with cytochalasin B during the period of meiosis I (a) and meiosis II (b). Usually only polar bodies developing at meiosis II possess chromatin that becomes organized into a nucleus. (a) Nucleus (N) in a polar body formed after inhibition of the first polar body. (b) Chromatin of polar bodies that form at the first meiotic division may be surrounded by cisternae (arrows), however, and a nuclear envelope usually fails to form. Scale bar = 1 μm.

crotubule dynamics in CB-treated eggs (see below) are consistent with this notion.

Sperm nuclear transformations

The different rates of sperm nuclear expansion observed here are similar to those that have been previously reported in oysters (Kuraishi and Osanai, 1988; Komaru *et al.*, 1990) and in other mollusks (Da-Yuan and Longo, 1983; Luttmer and Longo, 1988; Longo and Scarpa, 1991). It is noteworthy that during polar body formation in eggs inseminated at an arrested stage of meiosis there is a condensation of the dispersed sperm chromatin (Longo, 1990). Such a size reduction was not noted here, due possibly to the heterogeneity of the population studied or to the presence of a relatively small decrease that went undetected. Inspection of micrographs from published investigations of fertilized oyster eggs reveals images of incorporated sperm nuclei that appear to have condensed following their initial dispersion (Kuraishi and Osanai, 1988; Komaru *et al.*, 1990).

It is not until the completion of meiosis and in conjunction with the assembly of a female pronucleus that a nuclear envelope formed along the periphery of the dispersed sperm chromatin. Nuclear envelope assembly with respect to both the male and female pronuclei involves an aggregation of cisternae along the periphery of the dispersed chromatin and their fusion (Lohka, 1988). The two pronuclei that form are virtually identical in size and structure; however, the female pronucleus is distinguished by its associated microtubule array and proximity to the polar bodies. The male pronucleus is identifiable by its association with the sperm aster. Morphogenesis of apposed male and female pronuclei of oyster zygotes is essentially the same as described for other mollusks (Longo and Anderson, 1969, 1970; Longo and Scarpa, 1991; see Longo, 1983).

CB effects

Results of the present study are consistent with previous investigations demonstrating that CB application leads to

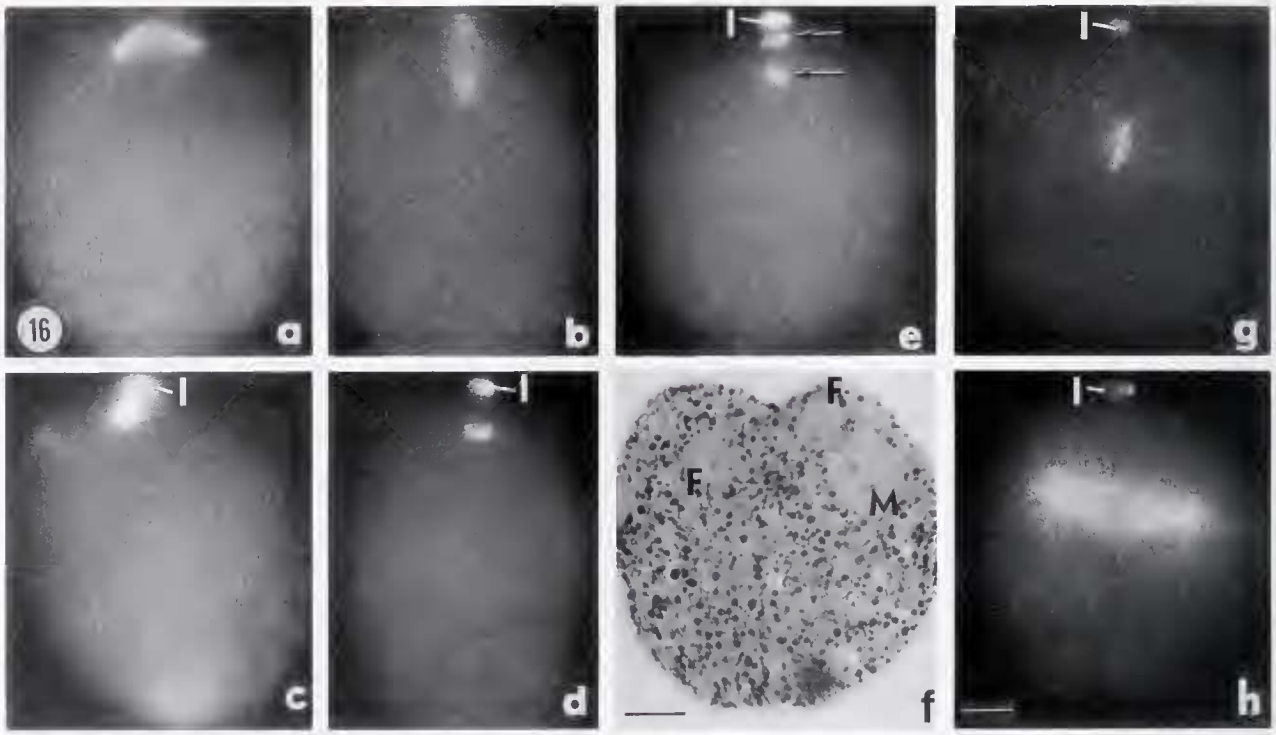

Figure 16. Effect of cytochalasin B (CB) on eggs at the second meiotic division. (a–d) When added immediately following first polar body formation, CB does not inhibit the development and changes in orientation of the second meiotic spindle. (a) A spindle forms that is initially positioned parallel to the egg surface; it then rotates to a perpendicular orientation (b) as in untreated eggs. (c, d) Same preparation shown in a and b, respectively, but viewed to show chromosomes stained with 7-AAD. (e, f) After spindle rotation, anaphase II ensues but is not accompanied by the formation of a second polar body. The chromosomes (arrows) retained within the egg develop into pronuclei (F, M), and the meiotic spindle regresses. (g, h) The two female pronuclei and the one male pronucleus that are formed undergo prophase and the chromosomes from all three pronuclei become organized on a single mitotic spindle. 1, first polar body. Scale bar = 10 μm.

polyploidy (Stanley *et al.*, 1981; Downing and Allen, 1987; Allen, 1987; Komaru *et al.*, 1990), particularly when given at two critical periods corresponding to anaphase I and II (Quillet and Panelay, 1986). As shown here and in previous studies with *Spisula* (Longo, 1972), CB has no apparent effect on the elevation of the cytoplasmic projection that becomes a polar body, but does affect its separation from the zygote. This is presumably a result of CB's action on the band of actin filaments comprising the cleavage furrow (Rappaport, 1975). Consequently, the projection that normally would become a polar body is not severed from the zygote, and chromosomes normally segregated from the zygote are retained. Depending on the time and duration of CB application, varying modes and degrees of ploidy result (Allen, 1987; Komaru *et al.*, 1990; Guo *et al.*, 1992a, b).

Recent investigations by Guo *et al.* (1992a, b) indicate that when CB is administered at metaphase I, greater than 50% aneuploids are produced. This result is in excellent agreement with the present study demonstrating that, when formation of the first polar body is blocked, chromosomes remaining in the zygote become organized on a tripolar, meiotic spindle. Orientation of the tripolar spindle and mechanisms by which it might function may be responsible for the distribution of aneuploidy around peaks with 23–27 and 35–37 chromosomes (Guo *et al.*, 1992a, b). The presence of a tripolar spindle is also consistent with investigations indicating that during meiosis there is a halving of material responsible for the organization of the meiotic spindle (Sluder *et al.*, 1989, 1993). Specifically, the observations presented here indicate that the spindle pole normally expelled with the first polar body is retained and, although it is unable to divide, it is able to participate in the organization of one pole of the tripolar spindle. In contrast, the center normally retained in the zygote following meiosis I divides and presumably is responsible for the organization of two poles of the tripolar spindle. This implies that the centrosome normally taken into the first polar body is affected in some way during the course of meiosis such that it is unable to be-

have in the same manner as the center normally retained within the zygote (Sluder *et al.*, 1989). These observations are consistent with recent experiments in starfish zygotes which demonstrate that the reproductive capacity of maternal centrosomes is degraded during meiosis I (Sluder *et al.*, 1993).

In theory, when only the first or the second polar body is blocked, two female pronuclei are formed; when both polar bodies are inhibited at least four female pronuclei are formed (Longo, 1972; Komaru *et al.*, 1990). Deviations from these values reflect the manner in which the second meiotic spindle becomes organized and the way the maternal chromosomes aggregate and become delimited by a nuclear envelope (Longo, 1972). In the treatments studied here, all the maternally derived pronuclei migrate to and become associated with the male pronucleus. The associated pronuclei then undergo prophase as separate structures, and their chromosomes become associated on a single mitotic spindle. Such an observation is consistent with previous accounts, indicating that centrosomes of the embryo are paternally inherited (Wilson, 1925; Sluder *et al.*, 1989, 1993).

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